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Extra-Telomeric Roles of Telomeric Proteins

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1. Introduction

Telomeres are tandem repeats of (TTAGGG)n sequence at the ends of chromosomes bound by a complex of proteins which are known to protect these ends from degradation and DNA repair activities. "Shelterin" is a dynamic multi-protein complex (Refer to previous chapters) (de Lange 2005) with DNA remodeling activity that acts together with several associated DNA repair factors to modify the structure of the telomeric DNA, thereby protecting chromosome ends (Bianchi et al. 1999; Griffith et al. 1999; Karlseder et al. 2002; Kim et al. 2003; Wang et al. 2011). While the main function of shelterin proteins is to bind and protect the telomeric repeats, telomere length per se is maintained and replenished by the ribonucleoprotein enzyme called "telomerase" which has a very specific TTAGGG repeat dependent reverse transcriptase activity (Blackburn 2000). Telomeres progressively shorten with each cell division in cultured primary human cells until a critically shortened length is reached, upon which the cells enter replicative senescence. Although the relevance of replicative senescence to aging in vivo remains poorly understood, numerous reports suggest that telomere shortening may be associated with organismal aging (Epel et al. 2009; Monaghan 2010), with concomitant metabolic decline and increased risk for disease and death (Jaskelioff et al. 2011; Jeon et al. 2011). Likewise, shorter telomere length has been shown to be associated with age-related diseases including coronary heart disease (Calvert et al. 2011; Krauss et al. 2011), hypertension (Dimitroulis et al. 2011), and dementia (Rolyan et al. 2011; Takata et al. 2011), as well as with a general risk for diseases such as insulin resistance and obesity (Al-Attas et al. 2010; Al-Attas et al. 2010; Makino et al. 2010; Njajou et al. 2011). However, the moleluar basis, if any, of these correlations and associations are not yet clear. Increasing amount of evidence suggests that telomeric proteins may also be involved in non-canonical activities at extra-telomeric sites or organelles within the cell. These novel, non-canonical functions may partially explain the mechanistic link between the telomere phenotypes found to be associated with physiological mechanisms, disease and organismal homeostasis (Teo et al. 2010; Gizard et al. 2011; Indran et al. 2011; Li et al. 2011; Martinez and Blasco 2011).

Since the discovery of telomeres, telomere-associated proteins have been thought to primarily function in telomere maintenance and homeostasis. Given that visualizing telomeric proteins by immunofluorescence techniques indicated that these proteins are exclusively present only at the telomeres gave rise to the notion that their function too is restricted at the telomeres. These conclusions gained further ground since the effect of loss of function of the telomeric proteins on organismal viability was also attributed entirely to

the telomere dysfunction. Yet the actual underlying mechanistic basis of the phenotypes exhibited by telomeric proteins have just started to unravel through interesting work by a number of laboratories. In this chapter, we will define some of the novel "extra-telomeric roles" of telomeric proteins since their functional locations and contexts are away from the telomere and/or they are independent of the telomere length or status in the cell. We will discuss the studies that led to these novel roles, the discrepancies amongst the different observations and the evidence that these functions indeed are telomere-independent and finally the possible reason why these non-telomeric functions of telomeric proteins can have an effect on a number of human ailments.

2. Discovery of extra-telomeric roles of telomeric proteins

Although numerous studies have been carried out to elucidate protein-protein interactions and telomere localizations of telomeric proteins, the analysis of their subcellular and extra-telomeric localization and function has gained attention only recently (Takai et al. 2010). The shelterin components TPP1, Rap1 and POT1 were shown to be present in non-chromatin bound fractions of the cell (Takai et al. 2010). This has been driven by the discovery of extra-telomeric roles for members of the telomeric complex like TRF2 at DNA damage foci (Bradshaw et al. 2005; Kaminker et al. 2005) and TIN2 at HP1 marked extra-telomeric sites (Kaminker et al. 2005). Interestingly, several studies proposed recently that mammalian Rap1 is present in many subnuclear locations (Takai et al. 2010; Teo et al. 2010) which also suggested multiple roles of Rap1 away from the telomere. Notably, whereas neither the de Lange nor Blasco group found a significant role for Rap1 in inhibiting telomere fusions in mouse cells, a recent report by Baumann and colleagues suggests that this may be the case in human cells (Bae and Baumann 2007; Sarthy et al. 2009). The difference in these findings may be due to the species under investigation (i.e., mouse versus human) or the experimental approaches taken by each group. Indeed, the de Lange and Blasco groups used a loss of function approach whereas the Baumann group utilized ectopic expression of a fusion protein, which could account for the differences in the studies. Interestingly, while Rap1 was initially suggested to be stabilized in the nucleus by TRF2 in the murine cells, we and others have observed that in human cells Rap1 is stable in the cytosol even in the absence of TRF2 (Teo et al. 2010). We further discovered a novel role of Rap1 in regulation of the NFkB signaling cascade.

Elongation of chromosomal ends by telomerase(Blackburn et al. 1989) during cell division prevents senescence in cells (allowing cells to overcome the Hayflick limit (Hayflick 1965)). Consequently, telomerase activity in cancer cells is thought to assist in overcoming the replicative crisis by addition of telomeric repeats. It has been known for a while that telomerase activity is highly upregulated in a number of human cancers and that aberrant expression of telomerase is a common occurrence in a number of human diseases. It is assumed that elongation of telomeres is the primary function of the reactivated telomerase in human cancers (Counter et al. 1994; Shay and Wright 2005; Blackburn et al. 2006). However, various studies have highlighted novel roles for telomerase, which are independent of its function on the telomeres. Interestingly, a number of these roles are now being suggested to be important for the functional basis for the molecular function of telomerase's role in human cancers as well as diseases like atherosclerosis (Gizard et al. 2011). These functions include roles of telomerase in Wnt signaling (Park et al. 2009),

mitochondrial regulation (Gordon and Santos 2010; Indran et al. 2011; Mukherjee et al. 2011) and DNA damage response (Beliveau and Yaswen 2007; Chenette 2009; Tamakawa et al. 2010).

3. The multi-faceted roles of telomerase

Some of these novel, non-canonical functions of telomerase were initially described in murine studies (Gonzalez-Suarez et al. 2001; Artandi et al. 2002). It has been shown that TERT overexpression in mice leads to spontaneous tumorigenesis, although telomere length did not change appreciably (Blasco et al. 1996). However, murine telomeres are very long and pathologies related to telomere shortening and accelerated aging are only observed in fifth or sixth generation telomerase knockout mice (Blasco et al. 1997; Wynford-Thomas and Kipling 1997; Goytisolo et al. 2000; Herrera et al. 2000). Also, there is low-level telomerase activity in most somatic murine tissues including breast epithelia, a primary site of TERT overexpression mediated carcinogenesis (Greenberg et al. 1998; Artandi et al. 2002). Furthermore, recent studies in primary human mammary epithelial cells have identified an apparently telomere-independent function of hTERT that enables human mammary epithelial cells (HMECs) to proliferate in mitogen deficient conditions, a hallmark of cancer (Smith et al. 2003; Mukherjee et al. 2011). Another study demonstrates that overexpressing mTERT in skin epithelia causes proliferation of hair follicle stem cells (Sarin et al. 2005). This function was traced to mTERT's function as a co-factor on β-catenin containing transcriptional regulation of the Wnt signaling pathway (Park et al. 2009). Transgenic mice overexpressing TERT are susceptible to carcinogen induced tumor formation as well as increased wound healing. Ectopic telomerase overexpression protects cells from antiproliferative or apoptotic stimuli (Bodnar et al. 1998; Ren et al. 2001). Conversely, in a wide variety of cell types, telomerase inhibition can enhance sensitivity to cytotoxic drugs (Harley 2008; Joseph et al. 2010; Roth et al. 2010). Additionally, hTERT can function as a RNA dependent RNA polymerase that can bind to non hTR RNAs and mediate independent functions, especially in the mitochondria as well as regulate mitochondrial functions (Maida et al. 2009; Esakova and Krasilnikov 2010; Indran et al. 2011; Mukherjee et al. 2011; Nitta et al. 2011). This multitude of alternative functions of telomerase is suggestive of its broader role in regulating cellular physiology, especially in diseased states.

3.1 Role of telomerase in Wnt transcriptional control

The role of telomerase in cell growth and stem cell function has been well documented (Lansdorp 2008; Flores and Blasco 2010). By using an RNAi screen, Coussens and coworkers discovered HIF1α as a novel regulator of telomerase in murine embryonic stem cells, thereby hinting at a feedback mechanism that is relevant to stem cell survival in cancers (Coussens et al. 2010). Sarin et al. reported that forced overexpression of mTERT in mouse skin triggered hair follicles to enter or remain in the active phase (Sarin et al. 2005). The result was furry mice in which the normal regulation of hair growth was disrupted. Even more striking was the observation that this effect of mTERT overexpression occurred in mice lacking mTR (Sarin et al. 2005) and was supported by a catalytically inactive protein ($mTERT^{ci}$) (Choi et al. 2008), ruling out the possibility that mTERT's enzymatic role in telomere maintenance was responsible.

3.1.1 The Wnt signaling pathway

Wnt proteins are secreted morphogens that are required for basic developmental processes, such as cell-fate specification, progenitor-cell proliferation (Willert et al. 2003) and the control of asymmetric cell division, in many different species and organs(Lyuksyutova et al. 2003; Logan and Nusse 2004). Recently, its importance in embryogenesis and cancer (Lie et al. 2005) has gained attention. There are at least three different Wnt pathways: the canonical pathway, the planar cell polarity (PCP) pathway (Schlessinger et al. 2007) and the Wnt/ Ca2+ pathway (Miller et al. 1999). In the canonical Wnt pathway, the major effect of Wnt ligand binding to its receptor is the stabilization of cytoplasmic ß-catenin through inhibition of the ß-catenin degradation complex. ß-catenin is then free to enter the nucleus and activate Wnt-regulated genes through its interaction with TCF (T-cell factor) family transcription factors and concomitant recruitment of coactivators. Planar cell polarity (PCP) signaling leads to the activation of the small GTPases RHOA (RAS homologue gene-family member A) and RAC1, which activates the stress kinase JNK (Jun N-terminal kinase) and ROCK (RHO-associated coiled-coil-containing protein kinase 1) and this leads to remodeling of the cytoskeleton and changes in cell adhesion and motility (Malliri and Collard 2003; Hall 2005; Angers and Moon 2009; Hoogeboom and Burgering 2009). WNT-Ca2+ signaling is mediated through G proteins and phospholipases and leads to transient increases in cytoplasmic free calcium that subsequently activates the kinase PKC (protein kinase C) and CAMKII (calcium calmodulin mediated kinase II) and the phosphatase calcineurin. Calcineurin induces activation of transcription factor NFAT, which regulates ventral patterning(Komiya and Habas 2008). CamKII activates TAK1 and NLK kinases, which can interfere with TCF/ß-Catenin signaling in the canonical pathway (Miller et al. 1999; Komiya and Habas 2008; Dodge and Lum 2011).

3.1.2 Telomerase regulates Wnt signaling by associating with Brg1 to regulate beta-catenin dependent transcriptional targets

To identify genomic targets that might explain the effect of telomerase function observed previously in hair follicle stem cells (Sarin et al. 2005), gene expression changes following rapid downregulation of mTERT^{ci} in skin were monitored. Affected genes strongly correlated with those regulated by the Myc and Wnt pathways (Choi et al. 2008), and Park et al. demonstrated that telomerase ablation resulted in altered mRNA expression levels of genes involved in signal transduction and development of epithelia and cytoskeleton of mice (Park et al. 2009). They then analyzed, using pattern-matching algorithms, the genome-wide transcriptional response after modulating TERT levels in mice. The results of this analysis revealed that the transcriptional response for TERT was similar to that observed for Wnt and Myc signaling pathways. Similar to telomerase, both Myc (Wolfer and Ramaswamy 2011) and Wnt (Ramachandran et al. 2011) have critical roles in carcinogenesis, metastasis and stem cell biology (Reya and Clevers 2005; Smith et al. 2010). The ability of telomerase is therefore similar to the effect of ß-catenin in activating stem cells. Artandi et al then establish that TERT functions as a transcriptional co-factor for the Wnt/ß-catenin transcriptional complex. TERT achieves this function by specific interactions with BRG-1, an ATP-dependent SWI/SNF chromatin-remodeling factor that is required for ß-catenin transcriptional function. Consistent with this interaction, a T-cell factor (TCF)-binding site reporter construct (TOP-FLASH) was upregulated by overexpression of either mTERT or

mTERT^{ci} (where the catalytic subunit does not bind the RNA component TERC) in a BRG1-dependent, but mTERC-independent manner (Park et al. 2009), establishing that the enzymatic activity of telomerase is not required for this function. Although these results have the caveat that overexpression might create gain-of-function phenotypes, the results presented by Park and colleagues build a strong case for TERT mediated regulation of Wnt signaling. The authors provided evidences for specific binding of an epitope-tagged version of mTERT at Wnt-regulated promoters under conditions in which the protein was not overexpressed (Park et al. 2009). Furthermore, to demonstrate a role for TERT in Wnt pathway activation, they examined the consequences of TERT loss in three different contexts. In conditional mTERT knockout ES cells, basal and induced expression of the Wnt target genes like Axin2 was reduced upon mTERT excision. In another model, the impact of Xenopus TERT (xTERT) knockdown was examined in embryonic growth since Wnt signaling is important during Xenopus laevis development (Lyuksyutova et al. 2003). The injection of two different morpholinos directed against xTERT into frog embryos caused striking defects in anterior-posterior axis formation. These defects were rescued by co-injection of morpholino-resistant xTERT or xTERTci mRNAs, thereby suggesting that these effects are specific and due to a noncatalytic role of TERT. Remarkably, first-generation mTERT-deficient mice (which still have long and functional telomeres), while superficially normal, revealed a partially penetrant (~50% of animals) homeotic transformation of the vertebrae, observed as loss of the 13th rib on one or both sides, proving the novel, extratelomeric role for TERT dependent Wnt signaling during development.

3.2 Role of telomerase in DNA damage repair

It has been shown that telomerase, together with other telomere-specific binding proteins, maintains telomere heterochromatin, thereby preventing telomere degradation and the activation of the DNA damage response pathway (Chan and Blackburn 2002). DNA repair proteins (such as ATM, WRN, MRN complex) have been found to play essential roles in the telomere maintenance (Machwe et al. 2004; Blasco 2005; de Lange 2005; Burma et al. 2006; van Overbeek and de Lange 2006). Prior work in both budding yeast (Flint et al. 1994; Myung et al. 2001) and mammalian cells (Sprung et al. 1999; Stellwagen et al. 2003; Gao et al. 2008) indicates that chromosome breaks at locations distinct from telomeres are occasionally repaired by telomere addition; however, this mechanism for chromosome healing occurs much less frequently than other forms of DNA repair. However, it is also possible that telomerase may participate in other forms of DNA repair. This was elegantly shown in a study by Masutomi and coworkers, which implicates hTERT as a regulator of the DNA damage response pathway and in regulating histone dependent chromatin reorganization (Masutomi et al. 2005).

3.2.1 DNA double strand break repair pathways

DNA double-strand breaks (DSBs) pose a serious threat to cell viability and genome stability. Under homeostasis, DSB are generated during replication by blocking lesions resulting from reactive oxygen species (ROS) leading to fork collapse; during genomic rearrangements such as yeast mating-type switching, V(D)J recombination, class-switch recombination, meiosis; and from physical stress when dicentric or catenated chromosomes are pulled to opposite poles during mitosis (Franco et al. 2006; Keeney and Neale 2006;

Chaudhuri et al. 2007). DSBs are also produced when cells are exposed to DNA damaging agents including ionizing radiation (IR), which creates DSBs directly and indirectly via production of ROS; chemical agents and UV light that create replication blocking lesions (alkyl adducts, pyrimidine dimers, and crosslinks) e.g Doxorubicin; and cancer chemotherapeutics that poison topoisomerase I (which produces replication-blocking lesions) e.g. Camptothecin, or topoisomerase II, (which traps the enzyme-DNA complex) after DSB induction and can potentially produce DSBs during any phase of the cell cycle (Limoli et al. 2002; Furuta et al. 2003; Bryant et al. 2010). Failure to repair DSBs, or misrepair, can result in cell death or large-scale chromosome rearrangements including deletions, translocations, and chromosome fusions that enhance genome instability, a hallmark of cancer cells (Rothkamm et al. 2001; Degrassi et al. 2004; Su 2006; Puig et al. 2008; Sliwinska et al. 2009). Cells have evolved groups of proteins that function in signaling networks that sense DSBs or other DNA damage, arrest cell cycle, and activate DNA repair pathways (Franco et al. 2006). The cellular responses can occur at various stages of the cell cycle and are collectively called DNA damage checkpoints, but when cells suffer too much damage overlapping signaling pathways can trigger apoptosis to prevent propagation of cells with unstable genomes (Rothkamm et al. 2001; Franco et al. 2006; Su 2006; Jeggo and Lavin 2009).

Eukaryotic cells repair DSBs primarily by two mechanisms: nonhomologous end-joining (NHEJ) and homologous recombination (HR); also known as “error-prone” and “error-free” repair, respectively (Natarajan and Palitti 2008). Upon induction of a DSB, NHEJ proceeds in a stepwise manner beginning with limited end-processing by the MRE11/RAD50/NBS1 (MRN) complex, end-binding by Ku comprising the Ku70 and Ku80 subunits, and recruitment of the DNA-dependent protein kinase catalytic subunit (DNA-PKcs), forming the trimeric DNA-PK holoenzyme (Burma et al. 2006). Once bound to broken ends, DNA-PK is activated, consequently phosphorylating itself and other targets including RPA, WRN, and Artemis; in cells lacking ATM, DNA-PK can also phosphorylate histone H2AX, termed γ-H2AX (Stiff et al. 2004). In the final step, DNA ligase IV, with its binding partners XRCC4 and XLF, seals the break (Gu et al. 2007). An alternative Ligase III-mediated NHEJ pathway is promoted by PARP-1 and is more error-prone than classical NHEJ (Simsek et al. 2011). HR is considered a more accurate mechanism for DSB repair because broken ends use homologous sequences elsewhere in the genome (sister chromatids, homologous chromosomes, or repeated regions on the same or different chromosomes) to prime repair synthesis (Helleday 2003). With the exception of sister chromatids, repair templates are often not perfectly homologous, and in these cases HR results in loss of heterozygosity, with information transferred non-reciprocally from the unbroken (donor) locus to the broken (recipient) locus, a process termed gene conversion. HR initiates with extensive 5′ to 3′ end-processing at broken ends, which is regulated by MRN (Langerak et al. 2011). The resulting 3′ ssDNA tails are bound by hSSB (single-strand binding protein), which is replaced with Rad51 in a reaction mediated by various related proteins like RAD52, BRCA1 & 2, etc. (Qing et al. 2011). The resulting Rad51 nucleoprotein filament searches for and invades a homologous sequence, which can dissociate and anneal with the processed end of the non-invading strand on the opposite side of the DSB in a RAD54 dependent manner (Mazin et al. 2010), or both ends may invade producing a double-Holliday junction that is resolved to yield crossover or non-crossover recombinants. Thereafter, the remaining ssDNA gaps and nicks are repaired by DNA polymerase and DNA ligase (Lieber 2010).

3.2.2 Extra-telomeric role of telomerase in DNA damage repair

In an attempt to understand novel roles of telomerase, Masutomi et al analyzed the effects of suppressing hTERT function on the cells response to ionizing radiation. They examined previously characterized phosphorylation changes in several proteins of the DNA damage response (DDR) pathway in diploid human fibroblasts, which only transiently express low levels of hTERT in S-phase(Masutomi et al. 2003). Irradiation of human BJ or WI38 fibroblasts led to the phosphorylation of H2AX (γ-H2AX), ATM and BRCA1 proteins and up-regulated the p53 protein with similar results when treated with the chemotherapeutic agents irinotecan or etoposide. However, such treatments of these fibroblasts stably expressing either an hTERT-coding sequence-specific shRNA (hTERT shRNA) or an hTERT 3′ untranslated region-specific shRNA (hTERT 3′ UTR shRNA) to ionizing radiation, irinotecan, or etoposide failed to induce a similar degree of H2AX or ATM phosphorylation or stabilization of p53 protein. These findings indicate that in cells lacking hTERT, the DNA damage response is dampened. Conversely, the expression of WT hTERT, which is resistant to the effects of the hTERT 3′ UTR-specific shRNA in cells expressing this shRNA rescued telomerase activity and the DDR defect. They also found that fibroblasts expressing a catalytically inactive hTERT mutant (DN hTERT) as well as select other mutants that impair the catalytic activity of telomerase(Hahn et al. 1999), also showed an impaired DNA damage response, suggesting that chronic loss of hTERT function either by RNA interference or catalytic inhibition abrogates the cellular response to DNA damage, implicating hTERT as a critical regulator of the DNA damage response pathway. Interestingly, over the short time periods of these experiments (low population doublings), there were no detectable alterations in overall telomere length or changes in the length of the 3′ telomeric single-stranded overhang either before or after irradiation of cells expressing an hTERT-specific shRNA as compared with cells expressing a control shRNA. Furthermore, since they observed only 7% of nuclear foci containing γ-H2AX colocalized with telomeres after treatment with ionizing radiation, it is not possible to attribute the effects to a telomere specific effect. These observations are in line with a previous study, which demonstrates that during senescence in human cell culture or in mice, there is an accumulation of irreparable DNA damage lesions (Sedelnikova et al. 2004). They further show that hTERT ablation reduced ATM autophosphorylation in response to ionizing radiation, as well as to agents such as trichostatin A (TSA). These findings suggest that suppression of hTERT expression modulates overall chromatin architecture. Therefore, they went on to demonstrate that chromatin derived from cells lacking hTERT was slightly more susceptible to MN (micrococcal nuclease) digestion, particularly at earlier time points compared with control cell lines. Remarkably, they found decreased levels of histone H3-lysine (K) 9 dimethylation and increased amounts of H3-K9 acetylation in cells lacking hTERT. The heterochromatic proteins 1 (HP1) associate with di- and tri-methylated but not to acetylated forms of H3-K9 to form heterochromatin (Murr 2010). Supportive of this observation, they also demonstrate by acid extraction of histones, that H2Ax levels in soluble fractions were higher than the chromatin bound insoluble fraction in hTERT ablated cells compared to controls. However, there were no appreciable differences in the amounts of soluble macro H2A.1, H2B, H3, and H4 in cells that expressed or lacked hTERT expression, suggesting that it is the stress dependent chromatin remodeling that is dependent on TERT. This is experimentally proven by altered migration of chromatin in pulsed-field gel electrophoresis.

Telomerase knockout mice lacking the RNA component mTerc also show impaired responses to agents that damage DNA (Wong et al. 2000); although the effects are apparent only in late generation mTerc-null mice that show significant telomere shortening and dysfunction (Goytisolo et al. 2000). Recent studies however report that there are no detectable effects of mTert or mTerc deficiency on chromatin structure and DNA damage responses (Vidal-Cardenas and Greider 2010). Although, regulation of chromatin plays a critical role in mammalian development(Meehan 2003; Kim et al. 2009; Cheng and Blumenthal 2010), developmental compensation in murine genetic models or species differences may account for the differences observed in the studies. Such developmental compensation, as has been observed in mice lacking the retinoblastoma gene (Sage et al. 2003), might similarly mask the effects of germ-line telomerase loss on chromatin structure and DNA damage.

Telomerase is reactivated in 80-90% of all cancers, providing the cancer cells with increased proliferative and survival properties (Harley 2008). Mouse tumors also show evidence of increased telomerase activity (Blasco et al. 1996), despite harboring long telomeres and basal telomerase activity. Because recent work in both transgenic mice and human cells suggests that increased TERT expression contributes to malignant transformation even in cells harboring long telomeres (Blasco and Hahn 2003; Blasco 2005; Jones et al. 2011), the effects of hTERT on chromatin may provide a plausible mechanism for such additional functions of hTERT in maintaining chromosomal stability and may suggest how TERT can contribute to cell transformation independently of its effects on telomere maintenance.

3.3 Role of telomerase in mitochondria

3.3.1 Mechanisms of oxidative-stress

Tissue homeostasis is controlled by the balance between the rates of cell proliferation and cell death, which in turn is tightly regulated by diverse intracellular signaling cascades. It has been shown that the intracellular redox milieu which is driven by a balance between rate of generation of intracellular reactive oxygen species (ROS) and the efficiency of their scavenging play a critical role in controlling cellular signaling, protein turnover, ion transport and ultimately cell survival. Abnormal accumulation of ROS contributes to cellular aging and the senescence process whilst the ability to withstand oxidative stress leads to enhanced longevity in several species (Finkel and Holbrook 2000; Ma 2010). Although telomere length and its attrition contribute to senescence, telomere attrition is not the only stimulus for replicative senescence. A growing body of evidence suggests that oxidative stress can induce or accelerate the onset of replicative senescence, a phenomenon referred to as stress-induced replicative senescence (Toussaint et al. 2000; Duan et al. 2005). Interestingly, depending upon intracellular levels and the nature of the ROS species, the effects could be as diverse as activation of gene transcription and proliferation to DNA damage and cell death induction (Valko et al. 2006; Valko et al. 2007), which is intimately correlative to associated diseases phenotypes. The correlation between a "pro-oxidant" state and cell survival has been further consolidated by findings using oncogene-induced models of cell transformation, such as activated Rac, Bcl-2, and Akt/PKB (Clement et al. 2003; Lim and Clement 2007; Ma 2010).

Along with membrane bound NADPH oxidase complex (Nox family), the mitochondrial electron transport chain (ETC) also serves as a major intracellular source of ROS. The high flux of O_2 through ETC (almost 90% for oxidative phosphorylation) is accompanied by

leakage of electrons, resulting in the generation of superoxide anions, predominantly involving complexes I and III of the ETC (Vercesi et al. 2006; Orrenius et al. 2007). The presence of Mn superoxide dismutase (MnSOD; SOD2) in the mitochondrial matrix coordinates with GSH to scavenge these superoxide anions, thereby limiting the excessive accumulation of H_2O_2. The cytosolic Cu/Zn SOD (SOD1) serves a similar function in scavenging ROS generated outside of the mitochondria or directly released from the mitochondria. Therefore, efficient shuttling of electrons across the ETC coupled with mitochondrial O_2 consumption is important for normal mitochondrial physiology including ATP synthesis. In addition to mitochondrial DNA damage, excessive ROS accumulation in the mitochondria could trigger mitochondrial outer membrane permeabilization and facilitate the egress of apoptosis amplification factors such cytochrome C, apoptosis inducing factor (AIF), and Smac/Diablo.

3.3.2 Effects of telomerase on oxidative stress

TERT expression itself is regulated by many transcription factors, including AP1, SP1 and NF-κB, all of which are redox regulated (Poole et al. 2001; Akiyama et al. 2003; Takakura et al. 2005; Cong and Shay 2008). AP1 has been shown to suppress hTERT expression whilst SP1 and NF-κB are strong activators of TERT. Furthermore, prevention of nuclear export of hTERT enhances the anti-apoptotic activity of TERT against ROS-dependent apoptosis (Haendeler et al. 2004; Kurz et al. 2004).

Extra-nuclear hTERT has been shown to translocate to the mitochondria following oxidative stress (Santos et al. 2004; Santos et al. 2006; Kovalenko et al. 2010) however, its mechanistic role in mitochondrial function only emerged recently (Kovalenko et al. 2010; Indran et al. 2011; Mukherjee et al. 2011; Nitta et al. 2011). Dairkee et al. suggested a signature pattern characteristic of tumor cell immortalization (immortalization signature or ImmSig) wherein overexpression of oxidoreductase genes was identified as an important marker (Dairkee et al. 2004). As expected, hTERT ablation reversed ImmSig expression, increased cellular ROS levels, altered mitochondrial membrane potential, and induced apoptotic and proliferative changes in immortalized cells (Dairkee et al. 2004). While certain reports suggested that the localization of hTERT to the mitochondria resulted in increased mitochondrial DNA damage following acute oxidative stress, hTERT expression did not alter the rate of H_2O_2 breakdown, although levels of chelatable metal ions were higher, suggesting that the damaging effects of hTERT could be attributed to the elevated levels of divalent metal ions via Fenton chemistry (Santos et al. 2004). However, other studies demonstrate anti-apoptotic effects of mitochondrial localization of hTERT (Massard et al. 2006; Cong and Shay 2008) suggesting that Bax oligomerisation on mitochondrial membranes could be hTERT regulated. Several recent studies confirmed the protective response against oxidative stress in hTERT expressing cells (Massard et al. 2006; Mondello et al. 2006; Ahmed et al. 2008; Lee et al. 2008; Haendeler et al. 2009) and indicated that the function at the mitochondria is independent of its telomere synthesis activity.

3.3.3 Mitochondria specific functions of telomerase

Ahmed et al report that telomerase expressing MRC-5 lung fibroblasts demonstrated a significant translocation of hTERT to the mitochondria following oxidative stress, which was followed by lower mitochondrial DNA damage, reduced mitochondrial peroxide levels

and an increase in mitochondrial membrane potential. In addition, reports have also suggested that hTERT expression positively impacts mitochondrial function by improving its calcium buffering capacity and reducing O_2^- production (Massard et al. 2006). Haelender et al demonstrate that mitochondrial hTERT exerted a novel protective function by binding to mitochondrial DNA, increasing respiratory chain activity, and protecting against oxidative stress-induced damage (Haendeler et al. 2009). Studies also suggest that hTERT expression in cells improves mitochondrial function by maintaining mitochondrial membrane potential, improving calcium storage and reducing mitochondrial formation (Massard et al. 2006; Passos et al. 2007; Ahmed et al. 2008). Microarray profiling of gene expression in cancer cells established that telomerase activity controlled several glycolytic pathway genes, suggesting modulation of the energy state of tumor cells and telomerase ablation decreased glucose consumption, lactate production, cell proliferation and viability while bulk telomere length remained relatively unchanged (Li et al. 2005; Bagheri et al. 2006). In studies done by Haelender et al, mitochondria isolated from $TERT^{-/-}$ mice displayed a significant reduction in complex I dependent respiration as compared to mitochondria from TERT wild type mice. The study also identified two TERT binding regions in mitochondrial DNA containing the coding sequences for NADH:ubiquinone oxidoreductase (complex I) subunit 1 and 2 (ND1, ND2). They therefore hypothesized that mitochondrial TERT may counteract ROS production by complex I via binding to ND1 and ND2, thereby increasing the synthesis of functional complex I subunits, which in turn can reduce the formation of damaged complex I that leaks electrons onto oxygen. Indran et al reported that cells overexpressing hTERT have greater basal COX (cyclooxygenase) activity, suggesting improved mitochondrial efficiency leading to reduced ROS production (Indran et al. 2011). Therefore, hTERT may be regulating COX either by direct interaction, given hTERT's mitochondrial localization, or indirectly by binding to other COX interacting proteins such as Bcl-2. Alternatively, hTERT seems to modulate intracellular redox status by improving cellular antioxidant capacity via modulating glutathione levels in the cells and conferring survival advantages as evidenced by enhanced GSH/GSSG ratio in a variety of cell lines (Indran et al. 2011).

3.3.4 Mutational analysis of telomerase catalytic component hTERT decouples its functions at the telomere vs. mitochondria

Mukherjee et al studied a panel of hTERT mutants (Counter et al. 1998; Armbruster et al. 2001; Banik et al. 2002) and demonstrated that the ability of hTERT to enhance proliferation, which was a result of increased cell division and decreased apoptosis, could be genetically uncoupled from its functions in telomere elongation, lifespan extension, and DNA damage responses. Remarkably, they identified telomere elongation-deficient mutants that were still able to extend cellular lifespan. Loss of this specific ability was seen upon hPOT1 ablation, suggesting that the ability to recruit telomere-capping proteins is critical for the survival advantage of ectopic hTERT expression. They also discovered a novel role for telomerase in regulating mitochondrial RMRP (RNA component of mitochondrial RNA processing endoribonuclease) level (Maida et al. 2009) that was crucial for enhancing proliferation. They further demonstrated that the hTERT–RMRP pathway, which results in generation of siRNAs and feedback suppression of RMRP, is linked to the enhanced cell proliferation phenotype in HMECs (human mammary epithelial cells). RMRP has varied cellular functions, including processing RNAs required to generate primers for mitochondrial DNA replication, pre-rRNA processing during rRNA maturation, mRNA cleavage of cell cycle

genes, and potential regulation of gene expression via complexing with hTERT to generate siRNAs (Maida et al. 2009; Esakova and Krasilnikov 2010). These results therefore provide critical genetic support for the idea that hTERT has at least four biological functions—regulation of cell survival/proliferation, telomere elongation, cellular lifespan extension, and regulation of DNA damage responses. Interestingly, these studies concluded that all the diverse biological roles of hTERT require hTERT catalytic activity, even though all but telomere elongation are functionally independent of telomere. This observation differed from that observed by Park et al., in Wnt signaling (previous section) where the TERC was dispensable. One hTERT mutant that was predominantly cytoplasmic localized but retained catalytic activity was unable to enhance proliferation, indicating the requirement for nuclear localisation and decoupling its function in mitochondria. This observation has importance in diseases like CHH (cartilage hair hypoplasia), a pleiotropic disorder characterized by a short stature with other skeletal abnormalities, hypoplastic hair, immune deficiency and neuronal dysplasia of the intestine (Ridanpaa et al. 2001). Patients with CHH show a predisposition to lymphomas and other cancers. Mutations in RMRP, encoding a structural RNA molecule, have been linked to CHH (Ridanpaa et al. 2001). The details of underlying mechanisms by which RMRP might impact the pro-proliferative effect of hTERT require further elucidation. Further investigation of these pathways, as well as of alternative hTERT activities and its other binding partners, would not only improve our understanding of how hTERT exerts its anti-ROS effects at the mitochondria but also suggest novel ways for the development of anti-telomerase cancer therapeutic agents.

4. Diverse roles of shelterin proteins

Shelterin is composed of six core members that include TRF1, TRF2 (Telomeric Repeat binding Factor 1 and 2), TIN2 (TRF1- and TRF2-Interacting Nuclear Factor 2), POT1 (Protection Of Telomeres 1), TPP1 (formerly known as TINT1, PTOP or PIP1) and RAP1 (Repressor/Activator Protein 1) (Broccoli et al. 1997; Smith et al. 1998; Ye and de Lange 2004; de Lange 2005; Xin et al. 2007; Kendellen et al. 2009; Abreu et al. 2010). Three members of this complex, TRF1, TRF2 and POT1, bind directly to telomeric DNA repeats and anchor the rest of the complex along the length of the telomeres. The actual length of the telomeres varies in various species and also within mammals. TRF1 and TRF2 bind the double-stranded (TTAGGG)n repeats of telomeres while POT1 binds to 3′ single-stranded G overhangs (Broccoli et al. 1997; Smith et al. 1998; Ye and de Lange 2004; de Lange 2005; Xin et al. 2007; Kendellen et al. 2009; Abreu et al. 2010). Rap1 was discovered as a TRF2 interacting factor and named such due to its sequence homology with the yeast Rap1 (Li et al. 2000). The yeast Rap1 has a multitude of functions both at the telomere and at other sites on the chromatin. These extra telomeric roles of yeast Rap1 include working as a transcription factor, and also participating in chromatin boundary formation and silencing at subtelomeric sites through the recruitment of Sir proteins (Morse 2000). Unlike the yeast Rap1 that can bind directly to DNA at telomeric and non-telomeric sites due to its two Myb domains, the human homolog contains a single Myb domain, which renders it unable to bind to DNA on its own. Hence, the mammalian Rap1 is recruited by its high affinity binding partner TRF2 to the telomeres. Mammalian Rap1 has been suggested to be required for telomere length maintenance (Li and de Lange 2003; O'Connor et al. 2004) and prevention of telomere recombination and fragility (Sfeir et al. 2009; Sfeir et al. 2010). Genetic abrogation of many of the shelterin complex components individually results in early embryonic lethality in

mice (Blasco 2005). The recent availability of several transgenic mouse models of shelterin complex components, as well as the generation of tissue-specific conditional mouse models of these components, has revealed a role for these proteins in cancer susceptibility and age related pathologies even in the presence of normal telomerase activity and under conditions when telomere length is normal (Blasco 2005). Recent work from different laboratories has suggested that Rap1 may not be directly involved in telomere protection, but rather in regulation of DNA repair activities at the telomere (Pardo and Marcand 2005; Bae and Baumann 2007; Sarthy et al. 2009; Bombarde et al. 2010; Sfeir et al. 2010; Chen et al. 2011). Sfeir et al have reported that conditional loss of Rap1 alone had little effect on telomere stability (i.e., no overt changes in telomere length, telomere DNA damage foci, or telomeric fusions were noted). However, when combined with loss of Ku, there was a marked increase in telomere sister chromatid exchanges, suggesting that Rap1 participates in inhibiting homology-directed repair (HR or HDR) at the telomere (Sfeir et al. 2010). Whole body or targeted depletion of Rap1 in stratified epithelia (in Rap1Δ/Δ K5-cre mice) does not affect the viability and fertility of the animals (Martinez et al. 2010; Sfeir et al. 2010). While skin specific deletion of Rap1 has been reported to cause both early onset of skin hyperpigmentation during adulthood and an obese phenotype in females (Martinez et al. 2010), there are no specific indications from these mouse models if Rap1 deletion has any role in longevity and cancer.

Clearly, the underlying molecular mechanisms by which telomerase and the shelterin complex proteins regulate processes critical for cancers like cell proliferation, survival upon genotoxic stress and invasion cannot be explained by the function of these proteins only on the telomere. Indeed recent studies have also indicated that telomeric proteins like Rap1 do have functional roles in diverse sub cellular locations (Martinez et al. 2010; Takai et al. 2010; Teo et al. 2010). In line with this, the expression levels of several other components of the shelterin proteins are altered in various human cancers (Blanco et al. 2007; Teo et al. 2010). These novel, non-telomeric roles of telomeric proteins could provide a useful Achilles' heel for developing drugs for a number of human ailments including cancer.

4.1 Rap1 in NFκB signaling

Using an unbiased screen we reported the identification of Rap1, as a novel adaptor of IKK and a critical regulator of NFkB. Our results documented the first, telomere independent, cytoplasmic role for mammalian Rap1 in transcription of NFkB target genes (Teo et al. 2010). We showed that Rap1 is part of the IKK complex and promotes IKK activity towards p65 subunit of NFkB by working as an adaptor (Teo et al. 2010).

4.1.1 The mediator of inflammation – NFκB (Nuclear factor κ-light chain enhancer of activated B cells)

Transcription factor NFkB is activated by a variety of cellular and developmental signals (Ghosh and Hayden 2008). Both activation and inactivation of NFkB signaling are rapid and tightly controlled events under normal physiological settings in most healthy cell types. Deregulated activation of NFkB has been observed and causally linked to development of several human pathologies including cancers (Ben-Neriah and Karin 2011). The NFκB family is composed of, RelA, RelB, c-Rel proteins and also includes the processed forms of p105 and p100 proteins, namely p50 and p52, respectively (Hayden and Ghosh 2008). All the Rel proteins contain a RHD (Rel Homology Domain), within which lies the DNA binding,

dimerization and IκB binding domains. The rate-limiting step in the activation of NFkB in response to stimulation is the degradation of the inhibitory IkB proteins that inhibit NFκB function mainly because they prevent the binding of NFκB dimers to DNA. IκBα, IκBβ, IκBε, IκBζ, IκBγ and BCL3 and the precursor p100 and p105 proteins are the identified members of the IkB family. Phosphorylation of IkBs by IkB kinases (IKK), IKK1 and IKK2, is critical for their subsequent ubiquitination and proteolytic degradation in response to stimulation(Hayden and Ghosh 2008; Ben-Neriah and Karin 2011). In particular, recent evidence has highlighted that IKK mediated covalent modification of free p65 subunit is critical for NFkB to function as an efficient transcription factor.

4.1.2 Cytosolic function of telomeric protein Rap1 in NFκB signaling

Gel filtration analysis of macromolecular complexes as well as direct biochemistry identified cytosolic Rap1 to be physically associated with the functional IKK complex, the primary node of NFkB activation. Biochemical experiments demonstrated that Rap1 was crucial for efficient recruitment of IKKs and phosphorylation of p65, an essential step for rendering NF-κB transcriptionally competent. Specifically, Rap1 was required in the IKK complex for it to be proficient in phosphorylating p65 on serine-536 in response to inflammatory stimuli. However, the phosphorylation and degradation of IkB was not significantly influenced in loss-of-function experiments. Rap1 was shown in kinase assays to be required by the IKK kinase complex to define specificity of the substrate; i.e., p65 compared to IkB or c-Rel. Moreover, the authors found that NF-κB signalling could in turn regulate Rap1 expression through NF-κB binding sites in the Rap1 promoter region. When NF-κB pathway activation was abrogated, total Rap1 expression was diminished; however, when the p65 subunit was overexpressed, more Rap1 was found in total cellular extracts, suggesting the existence of a feedback loop for Rap1 self-activation. In case of NFkB signaling, both the human and murine model are in agreement; Rap1 functions as an adapter that determines the substrate specificity of the IKK complex, thereby regulating inflammatory gene expression patterns (Teo et al. 2010). These findings also suggest the interesting possibility that Rap1 has additional partners, which stabilize it in various subcellular locations and that Rap1 functions, both in the nucleus (at the telomere and on chromatin) and in the cytoplasm. We further established the importance of this NFkB regulation in homeostasis by uncovering the effect of its dysregulation in cancer. Significantly, in human breast cancer tissue microarrays, we observed higher levels of cytosolic Rap1 corresponding with higher levels of activated NFkB, but also higher grades of tumor (Teo et al. 2010). These findings suggest that Rap1 and NFkB function in concert to enhance tumor survival and metastasis by upregulating NFkB dependent anti-apoptotic mechanisms.

4.2 Extratelomeric role of shelterin complex proteins Rap1, TRF1 and TRF2 in transcriptional control

Telomeric factors have long been known to play a role in binding at internal chromosomal locations. The first example of this kind was yeast Rap1, which specifically binds to telomeric DNA and was identified, at first, as a general regulatory factor. Interestingly, in yeast, telomere alterations can lead to the delocalization from telomeres of Rap1-associated heterochromatin factors that are able to operate at extratelomeric or interstitial genomic sites (Maillet et al. 1996; Marcand et al. 1996). Based on these yeast results, it is tempting to propose that telomeric factors are released from the telomeres after telomere shortening or

alteration and subsequently relocalize to extra-telomeric sites, where they modify the cellular transcriptional program.

In another evidence of a prominent non-telomeric transcriptional role of a telomeric protein, Martinez et al. performed chromatin immunoprecipitation sequencing (ChIP–seq) using RAP1 antibodies and discovered that it not only binds to telomeres but also to other non-telomeric sites throughout the murine genome, via recognition of the $(TTAGGG)_2$ consensus motif (Martinez et al. 2010). Extratelomeric RAP1 was shown to bind to non-coding regions in chromosomes 2, 11 and 17, which are enriched in TTAGGG tandem repeats, raising the possibility that RAP1 might prevent fragility and recombination at these genomic sites. Similar to its yeast counterpart, murine RAP1 binding sites were enriched at subtelomeric regions, leading to derepression of subtelomeric genes in Rap1-deficient cells, thereby indicating an evolutionarily conserved role for RAP1 in subtelomeric silencing. Although mammalian RAP1 was initially thought to have diverged from yeast Rap1 due to loss of its DNA-binding domain, these observations suggest that Rap1 may still be functionally conserved. The observation that RAP1 also binds to genomic sites lacking TTAGGG repeats and the fact that many of these sites were also associated with genes that are deregulated on Rap1 deletion suggests that RAP1 may interact with factors other than TRF2 to help gene transcriptional regulation. Gene set enrichment analysis (GseA) on genes downregulated in Rap1-null MEFs revealed significant downregulation of imprinted genes, as well as downregulation of genes involved in cancer, cell adhesion and metabolism (like peroxisome proliferator-activated receptor signaling, growth hormone and insulin secretion pathways) in Rap1-null cells. In turn, Rap1-null cells showed significant upregulation of ABC transporters and genes involved in type II diabetes, suggesting a negative effect of Rap1 deletion on metabolism. In agreement with a role of RAP1 in transcriptional regulation, RAP1-binding sites were shown to have RAP1-dependent enhancer activity. It should be noted that this Rap1 localization to various sites was not in response to any cellular stress. However, these data are slightly divergent from two subsequent reports also analyzed binding of telomeric proteins to extra-telomeric chromosomal sites by ChIP-seq (Simonet et al. 2011; Yang et al. 2011).

In the first study by Yang et al, analysis of genome-wide chromatin-binding patterns of two telomeric proteins RAP1 and TRF2 revealed that these proteins can associate with interstitial sites, too. In agreement with the study by Martinez et al, they demonstrate that TRF2 and Rap1 could bind distinct and overlapping extratelomeric chromatin binding sites (also called interstitial binding sites or ITS) that not only contained the TTAGGG repeat, but also non-telomeric repeat motifs. The binding overlap between Rap1 and TRF2 is expected given that these two proteins form stable protein heterodimers. In addition, they also identified unique bindings for RAP1 and TRF2, respectively, indicating TRF2-independent function of RAP1. The data suggest that while the binding of RAP1 to TTAGGG repeats could be TRF2 dependent, the mechanism by which RAP1 associates with non-telomere-repeat sequences could be dependent on its interaction with other chromatin-associated factors. Using stringent criteria, they predict ~ 300 potential TRF2 binding sites that contain telomeric repeats which was confirmed by TRF2 overexpression. They also observed that overexpression of TRF2 did not lead to an increase in the binding of endogenous RAP1 lending credence to the notion that extratelomeric binding of these telomeric proteins is not dependent on their interactions with each other.

A novel finding of the second study of TRF1 and TRF2 genome-wide binding by Simonet et al was the identification of non-ITS binding sites centered on (ATTCC)n satellite 2/3 repeats

or alphoid DNA satellite sequences, which form part of the most prominent autosomal heterochromatin blocks (Simonet et al. 2011). Given the reported role of TRF1 and TRF2 in the control of replication fork progression through telomeric chromatin(Sfeir et al. 2009; Ye et al. 2010), it is possible that these shelterin components play a similar role in other regions of DNA that are difficult to replicate, such as those packaged as heterochromatin. Gene ontology data (Simonet et al. 2011) suggested that a large subset of TRF binding sites may have functional relevance since they occur more frequently within or in close proximity to genes, which agrees with results from Martinez et al., since TRF sites are frequently located in intronic regions or distant from promoters(Simonet et al. 2011). Thus, TRF1 and TRF2 possibly regulate gene expression through looping mechanisms or by modifying the chromatin landscape. It is possible that cellular levels of TRF proteins influence their binding to the ITSs, and thus the expression of neighboring genes. Interestingly, they also observe from sequence alignments of bound and unbound extratelomeric sites that TRF1 and TRF2 discriminate between different sites on the basis of their length and sequence (Simonet et al. 2011). This observation suggests that other features such as accessibility and/or the chromatin structure of the surrounding DNA region may influence TRF binding. Thus, additional sites might be bound if the TRF protein concentration and/or chromatin context is altered.

However, in both studies in human cells (Simonet et al. 2011; Yang et al. 2011), the authors identified only a limited number of interstitial binding sites for RAP1, TRF2 and TRF1. Along those lines, it is possible that the number of RAP1 and TRF binding sites may have been underestimated, due to potential antibody-epitope sensitivity differences or due to species differences. Furthermore the general technical difficulty in identifying and calling peaks in repeat elements could be an additional reason for under-representation of binding sites. The data from Yang et al suggests that the cellular concentration of TRF2 may play a role in selective binding of TRF2 to its target sites. Since mouse telomeres are much longer than humans, it is plausible that differences in telomeric protein concentration may, at least in part account for the different binding site numbers observed between human and mouse.

4.3 Role of TRF2 in DNA damage repair pathway

Various laboratories have reported TRF2 association with proteins involved in DSB repair at the telomeres, including the MRE11/Rad50/NBS1 (MRN) complex, Ku70, WRN and BLM (Song et al. 2000; Zhu et al. 2000; Opresko et al. 2002; Dimitrova and de Lange 2009). Interestingly, Bradshaw et al demonstrate that TRF2 localizes to DSB (DNA double strand break; see description in previous section) sites at the early stages of cellular response to DSBs, appearing in the first few seconds after DSB induction and leaving as DSBs are being processed (Bradshaw et al. 2005). They created DSBs in defined nuclear regions of SV40-transformed human fibroblasts using pulsed laser microbeam irradiation followed by immunofluorescence monitoring of TRF2, ATM and H2AX. These data indicated involvement of TRF2 in an ATM-independent DNA-damage response at extra-telomeric sites. This data also demonstrated that the N-terminal basic domain is essential for TRF2 to associate with nontelomeric DSBs in vivo in contrast to the requirement of both Myb and basic domains of TRF2 for telomere localization. Given earlier reports that TRF2 preferentially localizes to double-strand–single-strand junctions of artificial telomeres as opposed to related, blunt-ended DNA (Stansel et al. 2001), the data suggested that the TRF2 DSB response may be DNA structure dependent rather than sequence based. However,

these data were questioned by another study that could not find TRF2 localization to DSB (Williams et al. 2007). Yet, the differences in the studies could be attributed to differences in the method of inducing DSB and the cellular context, indicating that the TRF2 response may be limited to specific kind and level of the damage.

A subsequent report also demonstrated that TRF2 is involved in DSB repair of non-telomeric DNA (Mao et al. 2007). Their results corroborated earlier findings that TRF2 represses NHEJ (non-homologous end joining) but show that it is required for HR (homologous recombination). TRF2 stimulated HR at nontelomeric DSB as demonstrated by ectopic expression of the full-length TRF2 and C-terminally truncated TRF2 ($TRF2^{\Delta M}$). The N-terminal domain of TRF2 has DNA binding activity independent of the TTAGGG sequence (Fouche et al. 2006). They also show that the TRF2 mutant ($TRF2^{\Delta M}$) that contains this unspecific DNA-binding domain and lacks the C-terminal domain stimulates HR when overexpressed (Mao et al. 2007). Thus, it is likely that the N-terminal domain of TRF2 is involved in strand invasion during HR repair of DSBs, and the C-terminal domain plays a regulatory role by limiting the HR activity of TRF2. Conversely, depletion of TRF2 strongly inhibited HR evidenced by delayed Rad51 foci formation after irradiation without affecting NHEJ. These results suggest that TRF2 plays a functional role in HR and may inhibit NHEJ by directing DSB repair toward HR pathway. Although TRF2 inhibits HR at telomeres, the observation that it mediates formation of T-loop formation at telomeres (Stansel et al. 2001), which resemble the structure of Holliday junctions is indicative of a role in HR. The basic domain of TRF2 binds to Holliday junctions in a sequence-unspecific manner (Fouche et al. 2006) suggesting that TRF2 may participate in HR of nontelomeric DNA.

Interestingly, since depletion of TRF2 delays Rad51 foci formation after irradiation by ≈4 h, it is speculated that TRF2 is required to directly recruit Rad51 or its paralogs such as Rad52 and BRCA2. TRF2 may interact with these proteins and facilitate further recruitment of Rad51. Collectively these findings suggest that, based on the configuration of DNA ends or other factors, certain breaks are preferentially processed by NHEJ, and others are preferentially processed by HR. Since TRF2 is recruited to the site of DSB very early, within 2 sec of irradiation (Bradshaw et al. 2005), a possible scenario could be that TRF2 arrives early, marks certain DSBs for repair by HR, and facilitates strand invasion by Rad51. These data exemplify another distinct extra-telomeric role for TRF2 in regulation of the DNA damage repair pathways.

5. Conclusion

The discovery of varied novel extra telomeric functions of telomeric proteins may have critical implications not only for tumorigenesis and telomerase-targeted anticancer therapeutics but also for tissue regeneration, genetic disorders associated with defects in telomeric proteins, apart from normal biological processes such as tissue homeostasis and organismal aging. A key to realizing this potential lies in determining whether these extratelomeric functions are mediated by the same or different biochemical or molecular activities of these proteins and who their cellular partners are in such specific functions. This would not only help us understand how telomeric proteins perform these diverse roles, but also determine whether these different pathways could be selectively targeted to provide multiple independent therapeutic targets for distinct human ailments.

6. References

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